A Meeting of Two Chronobiological Systems: Circadian Proteins Period1 and BMAL1 Modulate the Human Hair Cycle Clock

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The hair follicle (HF) is a continuously remodeled mini organ that cycles between growth (anagen), regression (catagen), and relative quiescence (telogen). As the anagen-to-catagen transformation of microdissected human scalp HFs can be observed in organ culture, it permits the study of the unknown controls of autonomous, rhythmic tissue remodeling of the HF, which intersects developmental, chronobiological, and growth-regulatory mechanisms. The hypothesis that the peripheral clock system is involved in hair cycle control, i.e., the anagen-to-catagen transformation, was tested. Here we show that in the absence of central clock influences, isolated, organ-cultured human HFs show circadian changes in the gene and protein expression of core clock genes (*CLOCK*, *BMAL1*, and *Period1*) and clock-controlled genes (*c-Myc*, *NR1D1*, and *CDKN1A*), with Period1 expression being hair cycle dependent. Knockdown of either BMAL1 or Period1 in human anagen HFs significantly prolonged anagen. This provides evidence that peripheral core clock genes modulate human HF cycling and are an integral component of the human hair cycle clock. Specifically, our study identifies BMAL1 and Period1 as potential therapeutic targets for modulating human hair growth.

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INTRODUCTION

The hair follicle (HF) is a highly dynamic mini organ that undergoes a cyclical remodeling process called the hair cycle (Kligman, 1959; Paus and Cotsarelis, 1999; Stenn and Paus, 2001; Schneider *et al.*, 2009). In the hair cycle the HF cyclically undergoes massive cell death and subsequently regenerates, owing to its rich endowment with various stem cell populations (Lavker *et al.*, 2003; Cotsarelis, 2006; Fuchs, 2009; Plikus *et al.*, 2011, 2012; Plikus, 2012). It comprises three phases; the growth stage (anagen) is characterized by long-lasting epithelial proliferation and production of a pigmented hair shaft. Anagen is followed by rapid, apoptosis-driven organ involution (catagen) where the lower two thirds of the HF regress, and then by a phase of relative quiescence (telogen; Supplementary Figure S1 online). Because of its autonomous oscillatory behavior, the hair cycle represents an ideal model for studying complex mesodermal–neuroectodermal tissue interactions at the intersection of chronobiology, developmental biology, regenerative medicine, and systems biology (Stenn and Paus, 2001; Halloy *et al.*, 2002; Al-Nuaimi *et al.*, 2010, 2012; Plikus, 2012).

Although numerous molecular factors are known to have an impact on HF cycling, the basic controls of this oscillatory mechanism (''hair cycle clock'') remain unknown (Paus and Foitzik, 2004; Plikus *et al.*, 2008; Lin *et al.*, 2009; Schneider *et al.*, 2009). Investigating these controls is of major clinical relevance, as the vast majority of hair growth disorders can be attributed to altered HF cycling, in particular during the anagen–catagen transition (Paus and Cotsarelis, 1999; Cotsarelis and Millar, 2001; Paus and Foitzik, 2004; Paus, 2006; Peters *et al.*, 2006; Schneider *et al.*, 2009).

There is growing consensus that the regulatory mechanisms governing the human hair cycle are based on an intrafollicular oscillatory system (Robinson *et al.*, 1997; Paus and Foitzik, 2004; Kwon *et al.*, 2006; Lin *et al.*, 2009; Al-Nuaimi *et al.*, 2010, 2012; Plikus *et al.*, 2013). One such candidate is the circadian clock, a molecular oscillatory system with a 24-hour

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Abbreviations: CCG, clock-controlled gene; HF, hair follicle; MK, matrix keratinocyte; qRT–PCR, quantitative reverse transcriptase–PCR

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periodicity (Schibler and Sassone-Corsi, 2002; Dunlap *et al.*, 2004; Lowrey and Takahashi, 2004; Dardente and Cermakian, 2007; Miller *et al.*, 2007; Bass, 2012; Brown *et al.*, 2012; Feng and Lazar, 2012; Ota *et al.*, 2012; Plikus *et al.*, 2013; Supplementary Figure S2 online). The circadian clock is synchronized by the ''master regulator,'' the suprachiasmatic nucleus, which receives external cues, e.g., light and temperature, this leads to synchronization of the molecular clock found in peripheral tissues via sympathetic, parasympathetic, and glucocorticoid signals, although the exact mechanisms of this synchronization are not fully understood (Schibler and Sassone-Corsi, 2002; Dunlap *et al.*, 2004; Lowrey and Takahashi, 2004; Miller *et al.*, 2007; Sporl *et al.*, 2011; Bass, 2012; Brown *et al.*, 2012; Feng and Lazar, 2012; Ota *et al.*, 2012). More recently, there is increased evidence supporting the importance of peripheral clock activity on tissue function, separate from the suprachiasmatic nucleus, thus chronobiology research has entered into the field of peripheral tissue physiology (Dardente and Cermakian, 2007; Saini *et al.*, 2011; Sporl *et al.*, 2011; Albrecht, 2012; Ota *et al.*, 2012; Tonsfeldt and Chappell, 2012). As clock dysfunction may cause tissue pathology (Lee, 2005; Chen-Goodspeed and Lee, 2007; Takahashi *et al.*, 2008; Sahar and Sassone-Corsi, 2009; Geyfman and Andersen, 2010; Takita *et al.*, 2012; Geyfman *et al.*, 2012b), a greater understanding of the clock system and the ability to modulate it pharmacologically may have therapeutic benefits.

As cultured murine or human keratinocytes, fibroblasts, and melanocytes express clock genes and show 24-hour circadian rhythmicity (Kawara *et al.*, 2002; Tanioka *et al.*, 2009; Sporl *et al.*, 2011; Plikus *et al.*, 2013), and murine and human skin express clock genes (Zanello *et al.*, 2000), the molecular clock and clock-controlled genes (CCGs) may be implicated in human hair growth or cycle control (Supplementary Figure S2 online; Lin *et al.*, 2009; Geyfman and Andersen, 2010; Geyfman *et al.*, 2012a), in particular as deletion of core clock genes delayed anagen onset in mice (Lin *et al.*, 2009). In addition, clock genes impact cell cycle activity and apoptotic machineries (Fu *et al.*, 2002; Matsuo *et al.*, 2003; Lee, 2005; Chen-Goodspeed and Lee, 2007; Takahashi *et al.*, 2008; Sahar and Sassone-Corsi, 2009; Geyfman *et al.*, 2012b), which are key processes during HF cycling (Stenn and Paus, 2001; Paus and Foitzik, 2004; Schneider *et al.*, 2009; Al-Nuaimi *et al.*, 2012). Furthermore, clock genes co-ordinate the activation of murine HF stem cells (Janich *et al.*, 2011). Finally, plucked scalp hair shafts also permit one to study the human peripheral circadian clock (Akashi *et al.*, 2010).

On this basis, we hypothesized that clock genes (Dunlap *et al.*, 2004; Lowrey and Takahashi, 2004, 2011; Lee, 2005; Dardente and Cermakian, 2007; Saini *et al.*, 2011; Sporl *et al.*, 2011; Albrecht, 2012; Bass, 2012; Tonsfeldt and Chappell, 2012) may function as molecular components of the human ''hair cycle clock'' (Paus and Foitzik, 2004). To elucidate the role of the peripheral clock, in the absence of the central clock in human HFs, we have addressed two central questions.

1. Does the expression of clock genes or proteins in intact, isolated human scalp HFs, i.e., in the absence of central clock inputs, show circadian and/or hair cycle-dependent variations?
2. Does silencing core molecular clock components affect human HF cycling and hair growth *in vitro*?

RESULTS

Human anagen HFs transcribe core clock and CCGs with circadian rhythmicity

We first investigated whether the core clock genes, *CLOCK*, *BMAL1*, and *PER1* are transcribed in human anagen scalp HFs. As expected from previous data in murine and human skin, and plucked human hair shafts (Brown *et al.*, 2008; Akashi *et al.*, 2010; Geyfman *et al.*, 2012b; Sandu *et al.*, 2012), human anagen scalp HFs expressed CLOCK, BMAL1 and PER1 mRNA and protein (Figures 1, 2 and 3b, and Supplementary Figures S3 and S4 online). In addition, human anagen scalp HFs transcribed the CCGs, *c-Myc*, *NRD1* and *CDKN1a* (Figure 1).

Next, we determined by quantitative reverse transcriptase–PCR (qRT–PCR) whether human HFs also exhibit a circadian expression pattern for any of these genes. Following dexamethasone synchronization of clock gene activity (Balsalobre *et al.*, 2000), HFs were sampled every 4 hours over a 24-hour period (Figure 1b and c) or every 6 hours for 48 hours (Figure 1a). All three core clock genes and all tested CCGs (*NR1D1*, *c-Myc*, and *CDKN1a* (*P21*)) were expressed in the HFs of three separately tested patients, and showed circadian variation in their transcription patterns (Figure 1 a–c). Furthermore, Figure 1a shows that circadian rhythmicity was maintained over 48 hours. Despite the expected interindividual variation, all patients showed a similar rhythmicity over the test period, documenting that isolated human HFs exhibit peripheral molecular clock activity independent from central clock inputs.

Following this, HFs were cultured until half of them entered catagen, with the other half remaining in anagen (this took between 4 and 14 days). The time course was then repeated. This confirmed that there was circadian rhythmicity of clock gene expression, thus showing that the peripheral molecular clock was still active in both anagen and catagen HFs after 4 or more days in organ culture (Figure 3a).

CLOCK, PER1, and BMAL1 are also expressed at the protein level in human HFs

To better understand the functional role of the molecular clock in the human HF, clock protein expression was analyzed by immunohistochemistry. BMAL1 showed strong immunoreactivity in the matrix keratinocytes (MKs) of human anagen and catagen HFs (Figure 2a and b). BMAL1 protein was also located in the outer root sheath, dermal papilla, and connective tissue sheath (Figure 2a). Unlike BMAL1, PER1 protein immunoreactivity was restricted to the epithelium where it was most prominent in the outer root sheath (Figure 2c, d and f). CLOCK protein immunoreactivity was also restricted mainly to the HF epithelium, being more prominent in the outer root sheath than in the inner root sheath (Figure 2g).

Intrafollicular *PER1* gene and protein expression is hair cycle–dependent

To probe whether clock gene/protein expression in organ-cultured human scalp HFs is hair cycle dependent,

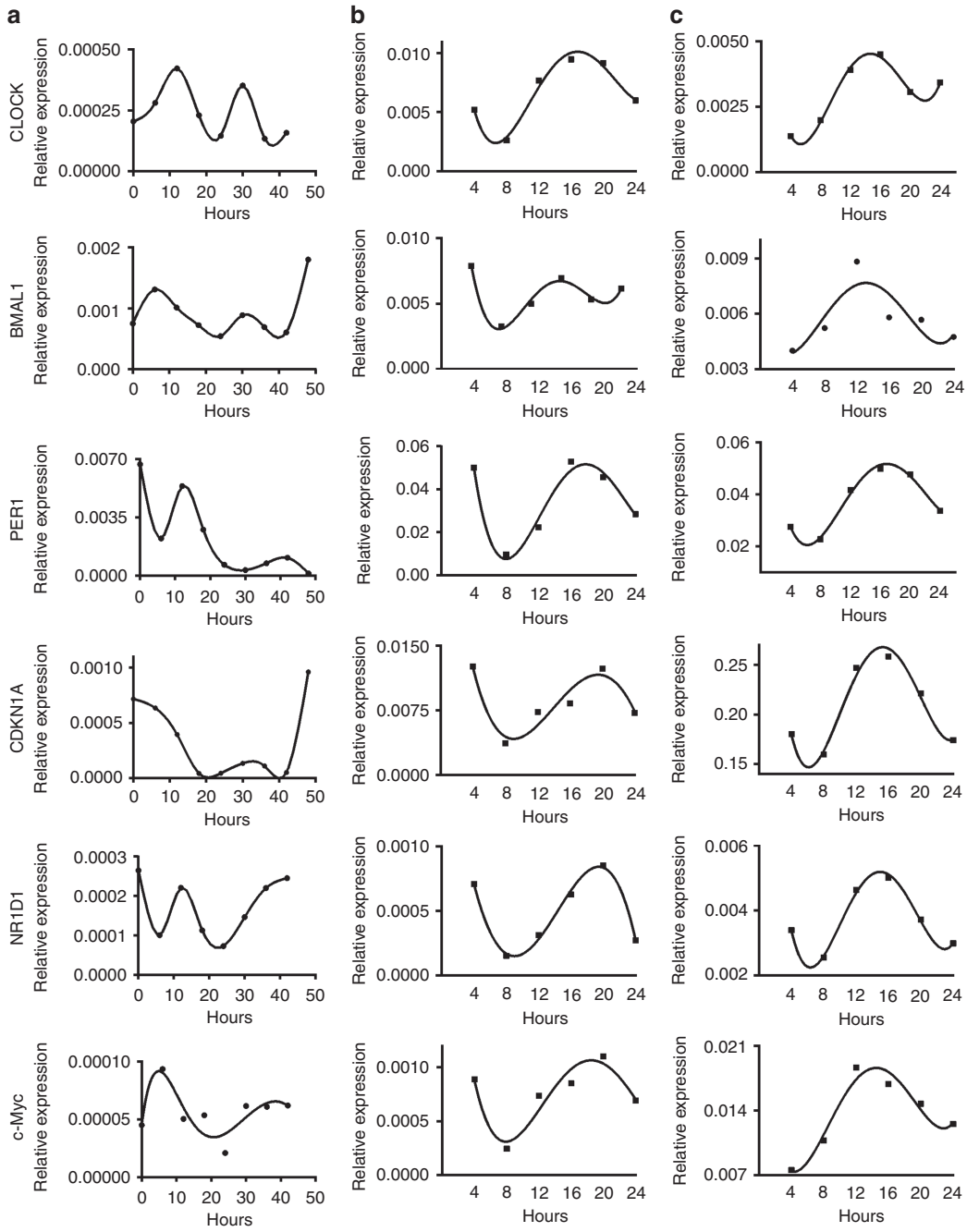

Figure 1. Circadian expression profiles of clock transcripts, *CLOCK*, *BMAL1*, and *PER1*, and clock-controlled genes, *NR1D1*, *C-MYC*, and *CDKN1A*, in isolated human anagen hair follicles. Transcript levels of the above candidates were quantified using quantitative reverse transcriptase–PCR in whole-hair follicles synchronized with dexamethasone and sampled for either 48 (**a**) or 24 hours (**b** and **c**) post synchronization. Data shown (black dots) are the mean relative expression levels of 15 hair follicles each from 3 different male individuals (**a**–**c**) compared with the housekeeping gene *PPIA*. All subjects showed circadian rhythmicity of all genes lasting 24 hours (**b** and **c**), which was further maintained for the full 48-hour time course (**a**). Data were not grouped because of recognized interindividual circadian variations between subjects (Akashi *et al.*, 2010).

anagen VI and catagen HFs were compared. This showed that the mRNA steady-state levels (Figure 3b) and the intrafollicular PER1 protein expression (Figure 2d and e) were significantly higher in catagen HFs compared with anagen VI HFs. In order to check whether the observed increase in PER1 expression was hair cycle dependent and did not result from diurnal expression changes, intrafollicular PER1 immunoreactivity was compared at two different time points (0900 and 1500 hours) in anagen and catagen HFs. This showed that there was no net change in diurnal PER1 expression irrespective of the time of day and that PER1 expression was consistently higher in catagen than in anagen HFs (Figure 2f). Furthermore, in synchronized HFs, the amplitude of *PER1* mRNA levels differed significantly between anagen

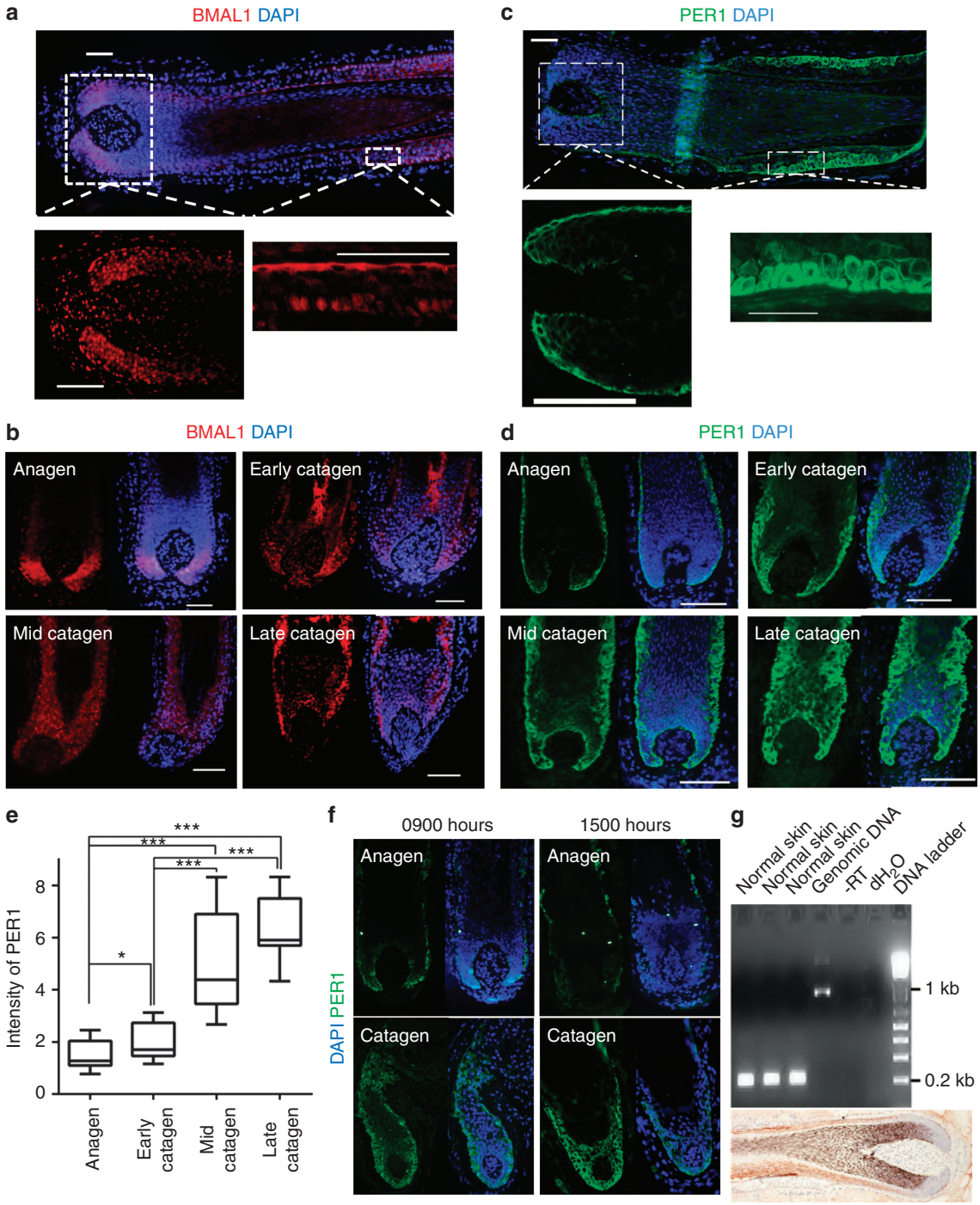

Figure 2. CLOCK, BMAL1, and PER1 expression in human hair follicles (HFs). (**a**) BMAL1 protein expression was found in the cell nuclei with high intensity in the hair matrix, dermal papilla, connective tissue sheath, and the outer and inner root sheaths, and did not show significant hair cycle-dependent expression changes (**b**). (**c**) PER1 protein expression was mainly cytoplasmic, localizing to the matrix keratinocytes and outer root sheath. (**d** and **e**) PER1 showed statistically significant hair cycle expression changes as human HFs progress from anagen VI through catagen (quantitative immunohistomorphometry, ImageJ). Mann–Whitney test (Holm–Bonferroni correction; $*P<0.05$, $***P<0.001$). (**f**) Diurnal expression changes were considered; however, PER1 showed no net change in protein expression over 6 hours. (**g**) CLOCK protein/mRNA levels were found by immunohistochemistry/reverse transcriptase–PCR in the HF localizing to the outer root sheath. Bar = 50 μm. DAPI, 4′,6-diamidino-2-phenylindole.

and catagen (Figure 3b). Although minor amplitude differences between anagen and catagen HFs were also seen for *CLOCK* and *BMAL1*, these did not reach statistical significance (Figures 2b and 3b). Taken together, this shows that the expression of at least one core clock gene product, PER1, is robustly hair cycle dependent.

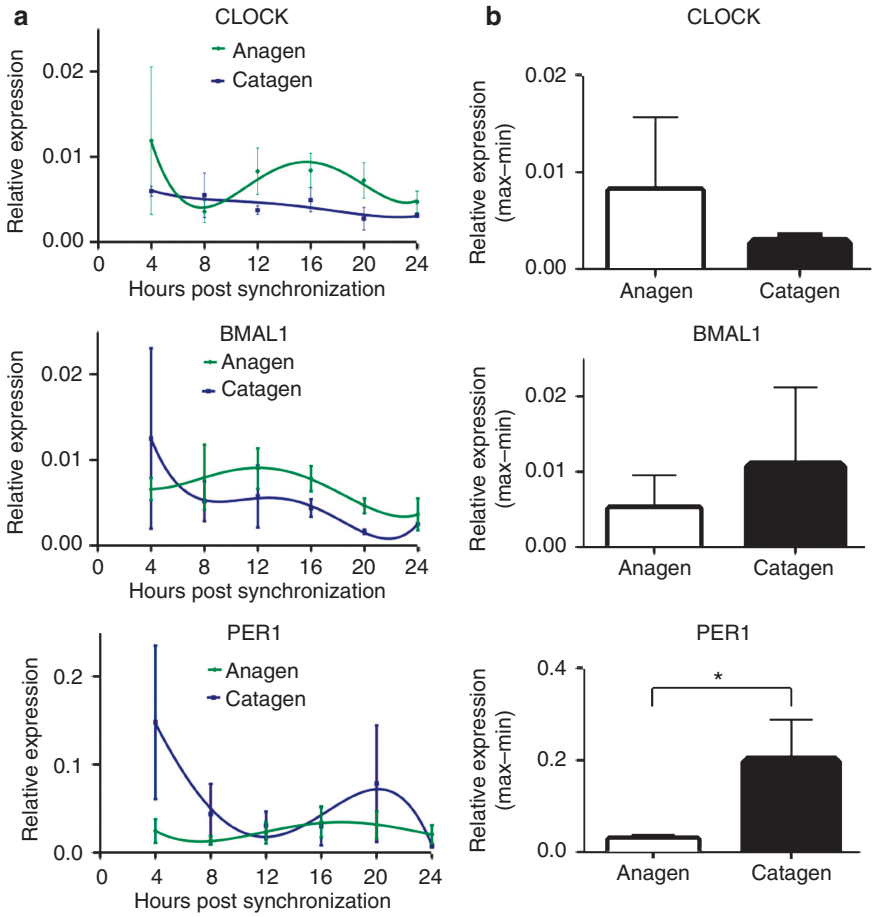

Figure 3. Time-series expression of clock mRNA in anagen and catagen human hair follicles (HFs). Human anagen VI HFs were cultured until half had spontaneously entered catagen (4–14 days); a time-course experiment was then performed. (**a**) Quantitative reverse transcriptase–PCR (five male patients) against the housekeeping gene *PPIA* showed that circadian rhythmicity was maintained beyond 4 days and *CLOCK* expression was significantly higher in anagen than in catagen ($P=0.046$). (**b**) On qualitative assessment, there was an apparent difference in waveforms; therefore, differences in amplitude between anagen and catagen were quantified (averaging maxima and minima expression), showing there was a statistically higher amplitude of *PER1* mRNA in catagen HFs (*indicates $P<0.05$, Student's *t*-test, ± SEM).

PER1 silencing in human HFs significantly prolongs anagen

Therefore, the functional consequences of reducing *PER1* gene activity on human HF cycling by intrafollicular gene knockdown was investigated (Samuelov *et al.*, 2012) by transfecting anagen VI HFs with *PER1* siRNA. Successful PER1 knockdown in human anagen HF organ culture was demonstrated at the mRNA and protein level (Supplementary Figure S4a online). Given that PER1 expression was low in anagen VI and sharply rose during catagen, we hypothesized that PER1 silencing would prolong anagen duration. Indeed, 96 hours after *PER1* knockdown, a significantly greater proportion of human HFs transfected with *PER1* siRNA had remained in anagen (71.4%) than in the scrambled oligo-treated control group (4.3%; Figure 4a). This observation was confirmed in four separate experiments from different individuals (Figure 4a). Hair cycle stage was confirmed by Ki-67/TUNEL staining (Kloepper *et al.*, 2010). Although there was a slight trend towards an increased number of proliferating (Ki-67-positive) cells in the MKs of PER1-silenced anagen VI HFs compared with a scrambled oligo control, this was not statistically significant. Nevertheless, this identifies PER1 as a catagen-inducing signal in human cycle control, whose silencing prolongs the duration of anagen.

BMAL1 or CLOCK silencing in human HFs also prolongs anagen

To assess whether anagen prolongation by PER1 silencing was PER1 specific or an effect of the peripheral core molecular clock, organ-cultured human HFs were transfected with a *BMAL1*-specific siRNA probe, which achieved knockdown on the mRNA and protein level (Supplementary Figure S4 online). This experiment was necessary, as BMAL1 is essential for the core clock oscillations to occur, it induces PER1 and its deletion eliminates clock activity (see Supplementary Figure S2 online), thus leading to disruption of the intrafollicular peripheral clock (Balsalobre *et al.*, 2000; Bunger *et al.*, 2000; Lee *et al.*, 2013).

Ninety-six hours after BMAL1 knockdown, a significantly greater proportion of silenced HFs (42%) remained in anagen VI than in the control group (10%; Figure 5a). Although BMAL1 silencing slightly modulated hair MK proliferation and

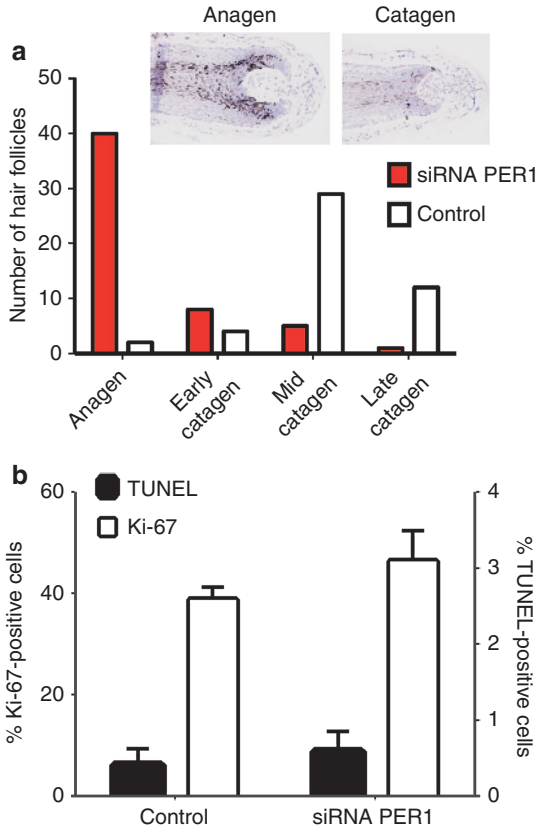

Figure 4. Effects of PER1 knockdown in human hair follicles (HFs). (**a**) Ninety-six hours post-PER1 knockdown, cycle stages were determined by morphology. A significantly higher number of HFs remained in anagen in silenced HFs ($P<0.05$, Fisher's exact test). (**b**) PER1 knockdown in HFs also increased proliferation (46.7%) 24 hours after transfection (assessed by Ki-67/TUNEL) compared with a control (36.0%). However, this was not statistically significant (Mann–Whitney, $P=0.2$). Error bars ± SEM. Results from four patients (three male/one female).

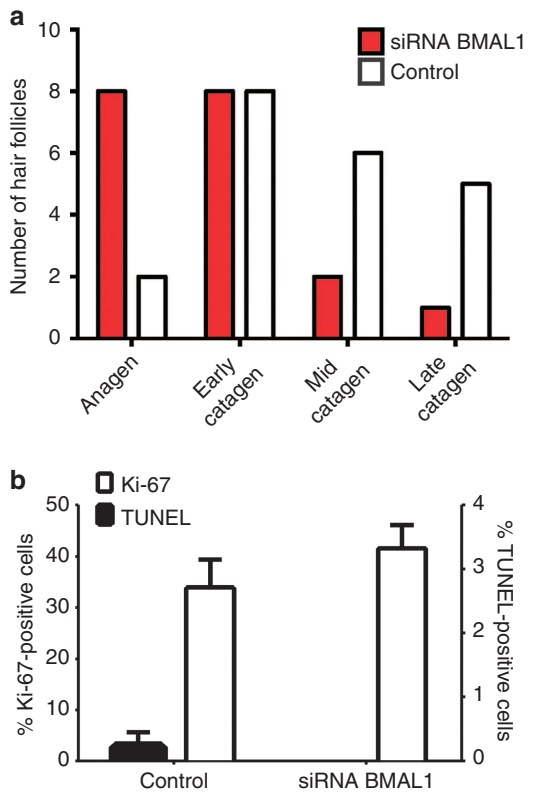

Figure 5. Effects of BMAL1 knockdown in human hair follicles (HFs). (**a**) Ninety-six hours post-BMAL1 knockdown, cycle stages were determined by morphology. A significantly higher number of HFs remained in anagen in silenced HFs ($P=0.028$, Fisher's exact test). (**b**) BMAL1 knockdown in HFs also increased proliferation (41.6%) 24 hours after transfection (assessed by Ki-67/TUNEL) compared with a control (33.9%). However, this was not statistically significant (Mann–Whitney, $P=0.29$). Error bars ± SEM. Results from three patients (two male/one female)

apoptosis, this did not reach significance. Pilot data from an additional CLOCK knockdown experiment (one patient) also demonstrated anagen prolongation (Supplementary Figure S5a online). Taken together, this suggests that the molecular clock as a system, rather than individual clock components, controls the human ''hair cycle clock''.

DISCUSSION

Following prior *in vivo* work in mice (Lin *et al.*, 2009) and human scalp hair shafts (Akashi *et al.*, 2010), to our knowledge our study provides the first evidence that intact human scalp HFs show both circadian and hair cycle-dependent clock gene activity in the absence of central clock influences. Moreover, we demonstrate that both peripheral clock PER1 and BMAL1 can regulate human HF cycling without input from the central clock. Specifically, we show that circadian activity is present after culture periods exceeding 4 days (Figure 3a) and, PER1 and BMAL1 produce anagen-terminating signals implicating their role in HF cycling (Figures 4 and 5).

Our findings correspond to a growing body of evidence that clock genes regulate physiological processes such as the cell cycle (Matsuo *et al.*, 2003; Khapre *et al.*, 2010) metabolism (Bass, 2012; Geyfman *et al.*, 2012b), tumor growth (Fu *et al.*, 2002; Chen-Goodspeed and Lee, 2007; Yang *et al.*, 2009), seasonal rhythms (Hazlerigg and Loudon, 2008), the reproductive cycle (Ware *et al.*, 2012), age-related pathologies such as Alzheimer's disease (Hatfield *et al.*, 2004; Bedrosian and Nelson, 2012), and other diseases including diabetes mellitus and depression (De Bodinat *et al.*, 2010; Etain *et al.*, 2011). The fact that CLOCK, PER1, BMAL1, and all CCGs studied show circadian rhythmicity lasting a minimum of 48 hours, beyond any transient effect of the dexamethasone synchronization (Balsalobre *et al.*, 2000), implicates their role in modulating the hair cycle. Thus, the autonomous oscillations of PER1 and BMAL1 observed in human scalp HFs support the importance of ''circadian'' clock functions in controlling local peripheral tissue physiology (Geyfman and Andersen, 2010; Janich *et al.*, 2011; Plikus *et al.*, 2011; Geyfman *et al.*, 2012b; Plikus *et al.*, 2013). Moreover, they suggest that the core peripheral clock is an integral component of the elusive ''hair cycle clock'' (Paus and Foitzik, 2004; Al-Nuaimi *et al.*, 2012; Supplementary Figure S1 online). Although murine *in vivo* work had already

implicated clock gene activity in the control of murine HF cycling (Lin *et al.*, 2009), our study shows that clock genes/proteins are expressed in human HFs exhibiting circadian rhythmicity and that the central clock is dispensable for clock-controlled hair cycle modulation.

Silencing both PER1 and BMAL1 had the same hair growth effects, which strongly suggests the importance of the core peripheral clock in human hair cycle control (*cf.* Supplementary Figure S2 online). This is corroborated by our CLOCK knockdown pilot data (Supplementary Figure S5a online). Although PER1 has many noncircadian roles in tumor suppression, cardiovascular disease, and Alzheimer's disease (Lee, 2006; Rosenwasser, 2010; Bedrosian and Nelson, 2012), as does BMAL1 in oxidative damage homeostasis and mitochondrial function (Knuever *et al.*, 2012; Razorenova, 2012), it could be argued that the effects observed are noncircadian and largely reflect a stress response. However, the fact that PER1, BMAL1, and CLOCK silencing all showed anagen-prolonging effects, while the HF's key response to stress is premature catagen induction (Schneider *et al.*, 2009) and that deletion of BMAL1 eliminates molecular clock activity (Bunger *et al.*, 2000; Lee *et al.*, 2013), suggests that the catagen delay is caused by the peripheral clock system.

The differences in expression of PER1 protein and mRNA between anagen and catagen reported here in human scalp HFs are mirrored in the murine hair cycle: *Per1* mRNA expression in mouse skin increases during the anagen–catagen transformation *in vivo* (Lin *et al.*, 2009), although less dramatically than during the human anagen–catagen transformation *in vitro*. Next, it was necessary to look at the diurnal effects of PER1 expression. Our results show that PER1 expression did not change over 6 hours, which was enough time to observe potential changes but too short for spontaneous catagen entry. The fact that PER1 expression was always higher in catagen regardless of the time of day shows that PER1 expression is regulated in a hair cycle-dependent manner.

In contrast to previous work our study is focused on the human system, and unlike previous human work clearly excludes the central clock. With our organ-culture model we are able to exclude any possible side effects of global clock gene knockout, species differences, and central inputs (Lin *et al.*, 2009; Akashi *et al.*, 2010). It is both a strength and limitation of this methodology that we can only draw conclusions on a functional role of the intrafollicular clock system in regulating the anagen–catagen transformation of human HFs *ex vivo*. The fact that recent microarray analyses of synchronized murine HFs show peripheral clock gene activity in other cycle transformation stages supports a role for the peripheral clock in influencing the 'hair cycle clock' (Lin *et al.*, 2009; Geyfman and Andersen, 2010; Geyfman *et al.*, 2012a; Plikus *et al.*, 2013), and suggests we would see similar effects if we were able to track the catagen–telogen transition or telogen–anagen transition in human HFs (see Supplementary Text ST2a online).

In human HFs, BMAL1 shows strong expression in the MKs. As BMAL1 has been linked with cell cycle control (Matsuo *et al.*, 2003; Sahar and Sassone-Corsi, 2009; Geyfman *et al.*, 2012b), this may be how it influences the hair cycle. As BMAL1 shows consistent protein expression throughout the anagen–catagen transformation (Figure 2b), differing from the murine system where *BMAL1* mRNA and protein expression peaked in late anagen, (Lin *et al.*, 2009; Plikus *et al.*, 2013), species–specific differences in the peripheral core clock on HF cycling may exist. The significant interindividual variations observed match earlier human work (Akashi *et al.*, 2010). However, our data show that the clock genes will continue to oscillate in human HFs in the absence of signals from the suprachiasmatic nucleus.

Although the mechanisms through which PER1 and BMAL1 exert their hair growth-modulatory effects remain to be dissected, they follow the established concept that clock genes and CCGs control cell cycling (Lowrey and Takahashi, 2004; Miller *et al.*, 2007; Geyfman *et al.*, 2012b; Plikus *et al.*, 2013). Recognized hair cycle-regulatory genes, *c-Myc* (Bull *et al.*, 2001; Bull *et al.*, 2005) and *p21* (Mitsui *et al.*, 2001; Ohtani *et al.*, 2007), are key cell cycle regulators and are reduced by PER1 knockdown. Moreover, the reduction of P21 by PER1 silencing corresponds well to the reduced p21 expression in BMAL1 knockout mice, which show delayed anagen onset (Lin *et al.*, 2009). PER2 has further been shown to control cyclin D1 (Fu *et al.*, 2002), a modulator of human HF cycling (Xu *et al.*, 2003). Thus, it is reasonable to hypothesize that both PER1 and BMAL1 regulate proliferation and apoptosis, and thus the anagen–catagen transformation by impacting the cell cycle and apoptotic machinery of MKs, similar to the role of CCGs Nr1d1 and Dbp in murine MKs (Lin *et al.*, 2009; Plikus *et al.*, 2013). Furthermore, PER2 mutations in humans cause familial advanced sleep-phase syndrome and are implicated in tumorigenesis and phase shifts in cyclin D1 and P21 (Gu *et al.*, 2012), whereas CLOCK mutations in humans leads to altered sleeping phenotypes (Wager-Smith and Kay, 2000). Although to the best of our knowledge there is no report of altered hair growth in such patients, subtle hair growth or cycling abnormalities may have been missed; therefore, future screening in such patients would provide definitive *in vivo* confirmation of our study in the future.

There is an increasingly appreciated link between the long-term effects of the clock and age-related pathologies. For example, reduction in circadian amplitude and response to external cues are linked with the severity of Alzheimer's disease, potentially by increased amyloid-β peptide accumulation (Hatfield *et al.*, 2004; Rosenwasser, 2010). This suggests that long-term clock disruptions lead to pathologies surpassing circadian boundaries (Rosenwasser, 2010). Our data raise the possibility that circadian clock outputs exert long-term cumulative effects. Specifically, clock protein accumulation within human HFs during anagen may represent one mechanism in which 24-hour rhythms impact the ''hair cycle clock'' and thus human HF cycling. However, with the work in peripheral clock biology in its infancy, validation of such a hypothesis would require further experimentation.

Evidently, a translational aspect of our study is that our results designate the peripheral core clock, specifically PER1

and BMAL1 activity, as promising targets for therapeutic hair growth modulation, e.g., with topically applied, HF-targeting (Chourasia and Jain, 2009; Liu *et al.*, 2011; Patzelt *et al.*, 2011) small molecule clock modifiers (Chen *et al.*, 2012), thus circumventing undesired effects on the central clock. Antagonizing the activity of PER1, BMAL1, CLOCK, and/or CCGs may counteract hair loss (alopecia and effluvium), whereas promoting activity of these targets may suppress unwanted hair growth (hirsutism and hypertrichosis; Cotsarelis and Millar, 2001; Paus, 2006).

In summary, our study supports the concept that the peripheral clock significantly modulates the anagen–catagen transformation of human HFs under clinically relevant *in vitro* conditions. We show that BMAL1, PER1, and, likely, CLOCK form an integral component of the human ''hair cycle clock.'' These clock genes, therefore, are targets for the therapeutic modulation of human hair growth. Moreover, we demonstrate that HF organ culture offers an instructive, clinically relevant model for preclinical peripheral clock research in a complex, oscillating human mini organ where two chronobiological systems meet.

MATERIALS AND METHODS

Human skin and HF collection

Redundant human scalp skin was obtained with written informed patient consent adhering to the Declaration of Helsinki Principles, from the temporal or occipital regions from females undergoing routine facelift surgery (total $n = 3$, 31–69 years) and scalp occipital HF units from males undergoing hair transplantation surgery (total $n = 10$, 28–48 years). Tissue was obtained following ethical and institutional approval (the University of Luebeck and the University of Manchester following human tissue act guidelines). Skin or HFs were fixed in 10% phosphate-buffered formalin, snap-frozen in liquid nitrogen or first embedded in Shandon Cryomatrix (Fisher Scientific, Loughborough, UK) before snap freezing.

Human HF organ culture

Human scalp HFs in anagen stage VI of the hair cycle (Supplementary Figure S1 online) were microdissected and organ cultured under serum-free conditions in the presence of insulin and hydrocortisone as described (Philpott *et al.*, 1990; Philpott *et al.*, 1994; Supplementary Text S1a online). Under these conditions, human anagen HFs continue to produce a pigmented hair shaft and eventually spontaneously enter a catagen-like state (Sanders *et al.*, 1994; Kloepper *et al.*, 2010). The telogen hair cycle phase cannot be captured in human HF organ culture.

Twenty-four-hour time-series experiment

Circadian rhythmicity of core clock genes and selected CCG expression was investigated in human anagen HFs from three male subjects (Supplementary Table S2a, b and c online). Microdissection and organ culture was started within 2 hours post surgery (time window of 0930–1300 h), and HFs were incubated for a 24-hour equilibration period. Clock activity was then synchronized (100 nM dexamethasone, 30 minutes; Balsalobre *et al.*, 2000), after which HFs were collected every 4 hours for 24 hours (patients b and c) or every 6 hours for 48 hours (patient a), and stored in RNAlater (Sigma, Surrey, UK) and then processed for qRT–PCR analysis.

In a second time-series experiment, HFs were cultured and staged according to macroscopic staging criteria (Kloepper *et al.*, 2010). For subject C (Supplementary Table S2 online), once half the HFs were in anagen and the others had entered catagen, the samples were synchronized and both anagen and catagen HFs were collected every 4 hours. For subjects D and E, this was repeated; however, all HFs entered catagen (Supplementary Table S2 online). This took between 4 and 14 days for the HFs to enter the correct stage. The HFs were maintained in RNAlater solution until processed for qRT–PCR.

Quantitative immunohistomorphometry

Immunohistochemistry or immunofluorescence microscopy staining for localization and quantification of clock proteins (CLOCK, BMAL1, and PER1) *in situ* was performed on human scalp (8 μm) skin or isolated HFs (6 μm; see Supplementary Table S1 online; Ackermann *et al.*, 2007) Primary antibodies were incubated overnight at 4 °C. Sections were washed in phosphate-buffered saline or TRIS-buffered saline between steps. Immunohistochemistry staining for Masson–Fontana and Ki-67/TUNEL double-immunofluorescence microscopy were carried out as previously described (Ito *et al.*, 2005; van Beek *et al.*, 2008; Kloepper *et al.*, 2010). Quantitative immunohistomorphometry in defined reference area, using standardized light exposure, was performed with Image J (NIH) software as described (Ito *et al.*, 2005; Kloepper *et al.*, 2010).

PER1 and BMAL1 knockdown in organ-cultured human HFs

Microdissected human anagen VI HFs were transfected with either *PER1* siRNA (PER1 FsiRNA (h): sc-38171; four subjects) or *BMAL1* siRNA (FsiRNA (h): sc-38165; three subjects) in organ culture, following the previously described Lipofectamine-based knockdown method (Chen and Roop, 2012), using scrambled oligo as a parallel control (see Supplementary Table S3 online for details). A pilot knockdown of a CLOCK supported this data (one subject; Supplementary Figure S5a online).

Quantitative reverse transcriptase–PCR

All qRT–PCR analyses for *CLOCK*, *BMAL1*, *PER1*, *NR1D1*, *c-Myc*, and *CDKN1A* were performed as described in the supplement, normalized to a housekeeping gene (*PPIA*; Supplementary Text S1b online and Supplementary Table S4 online).

In situ hybridization: *CLOCK* mRNA

Intrafollicular clock gene transcription was assessed by *in situ* hybridization, using digoxigenin-labeled *CLOCK* sense and antisense probes as previously described (Langmesser *et al.*, 2008 and see Supplementary Text S1c online for details).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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